[redacted]

SUBJECT: Abbreviated Risk Assessment for J-21 -0002-0003

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SUMMARY

The Agency has received a Microbial Commercial Activity Notice (MCAN) submission from [redacted] for two strains of *Saccharomyces cerevisiae*, [redacted] used for ethanol production. The subject strains are all derived from the commercially available *S. cerevisiae* [redacted] and were genetically engineered using various molecular biology techniques, including [redacted] strains were genetically modified to reduce formation of the [redacted] to the benefit of increasing ethanol yield per molecule of [redacted] In addition, strain [redacted] underwent further modifications to yield [redacted] a strain that can [redacted] in order to reduce the amount of commercial enzyme products containing [redacted] normally added during the simultaneous [redacted] phase of the ethanol production process (Cameron, 2021).

The genetic modifications to the recipient *S. cerevisiae* strain pose low concern for human health. The introduced genetic material does not pose pathogenicity, allergenicity, or toxicity concerns. No antibiotic resistance genes are present in the final production strain.

The genetic modifications to the recipient to arrive at the subject strains pose low ecological hazards. The introduced genes also do not provide a growth advantage relative to the recipient and there is a low probability of releases to the environment.

Therefore, there are low hazards posed by the use of both subject strains, *S. cerevisiae* strain [redacted] for ethanol production.

[redacted]

I. INTRODUCTION

The Agency has received a Microbial Commercial Activity Notice (MCAN) from DSM Bio-based Products and Services (Parsippany, NJ) for two strains of *Saccharomyces cerevisiae,* [redacted] and [redacted] to be used for fuel ethanol production. Both subject strains were genetically modified to reduce formation of the undesired byproduct [redacted] to the benefit of increasing ethanol yield per molecule of C6 sugar released from [redacted], such as [redacted] or [redacted]. In addition, strain [redacted] underwent further modifications to yield [redacted] a strain that can produce [redacted] in order to reduce the amount of commercial enzyme products containing [redacted] normally added during the [redacted] and [redacted] phase of the ethanol production process.

Although *S. cerevisiae* is one of the ten microorganisms eligible for the 5(h)4 Tiered Exemptions from MCAN reporting, and the submission claims that their strains meet all the criteria for the introduced genetic material for the Tier I Exemption, the company has chosen to submit these strains for an MCAN review because it is intended for use in, and thus transport to, multiple ethanol production facilities in the U.S. The transport of the microorganism to various facilities is outside the realm of the Tier I Exemption. As such, the company is submitting this MCAN for a thorough review of the intergeneric microorganisms given its use at a number of different ethanol production facilities, and thus removes the administrative burden of the Tier I application process from contracted manufacturers and ethanol producers.

The generic name for the two strains is "*Saccharomyces cerevisiae* modified."

II. TAXONOMY AND CHARACTERIZATION

The taxonomy and identification of the subject strains are described in detail in the Taxonomy and Identification Report (Rahman, 2021).

Saccharomyces cerevisiae has an extensive history of use in the area of food processing. Also known as baker's yeast or brewer's yeast, this organism has been used for centuries as leavening for bread and as a fermenter of alcoholic beverages. The risk assessment of *S. cerevisiae* for the 5(h)(4) Tiered Exemptions Final Risk Assessment for *S. cerevisiae:* (http://www.epa.gov/oppt/biotech/pubs/pdf/fra002.pdf) concluded that this yeast presents low hazards to human health and to the environment.

Although *S. cerevisiae* is associated with human activity from bread baking and fermentation of alcoholic beverages, *S. cerevisiae* is also widespread in nature. It has been recovered from a variety of sites such as soils, sediments, and plant material under different ecological conditions. *S. cerevisiae* is frequently recovered from fresh fruits and vegetables, generally those fruits with high levels of fermentable sugars. In the environment, yeasts can be dispersed by insects, particularly fruit flies (Gilbert, 1980). Becher et al. (2012) found that it is the yeast itself on fruits, and not volatiles given off from fruits, that attract fruit flies. In addition to its use in food processing, *S. cerevisiae* is widely used for the production of macromolecular cellular components such as lipids, proteins including enzymes, and vitamins (Bigelis, 1985; Stewart and Russell, 1985).

[redacted]

A. Parent and Recipient Microorganisms

The parental strain for this MCAN is *Saccharomyces cerevisiae* [redacted] a widely used commercial strain for ethanol production. The strain has been developed for the industrial ethanol industry and is available from [redacted], a division of [redacted] The strain was deposited into the submitter's internal collection under the designation [redacted] which refers to the same strain as in the commercial product [redacted] The strain has demonstrated high ethanol tolerance and is particularly well-suited for [redacted] etc.) and [redacted] (Fermentis product information sheet).

The MCAN describes the construction of the subject strains from *S. cerevisiae* [redacted] lineage. The subject strains were developed using synthetic biology, molecular biology, genetic engineering, and microbiological approaches, including transient antibiotic resistance.

The parent *S. cerevisiae* [redacted] is a well recognized industrial strain. The genome of this strain was sequenced and mapped by Wallace-Salinas et al. (2015). The authors reported a 99.2% similarity between [redacted]) and the reference *S. cerevisiae* strain [redacted] whose genome is available through the *Saccharomyces* Genome Database (www.yeastgenome.org). As reported in the MCAN, the genomes of the subject microorganisms [redacted] and [redacted] were sequenced and found to be identical to the parental *S. cerevisiae* [redacted]) sequence. In addition, all genes were functionally annotated with the software EggNOG-mapper (www.eggnog-mapper.embl.de) and 99% of the best hits have the closest match to *S. cerevisiae* proteins, which demonstrates that the species of the subjects and parent is *Saccharomyces cerevisiae*. The genome sequencing data are thus sufficient supplemental data to support the identification of the parent and subject strains.

<u>Taxonomy of the parental strain</u>

Name:	*Saccharomyces cerevisiae*
Class:	Saccharomyces
Order:	Saccharomycetales
Family:	Saccharomycetacae
Genus:	*Saccharomyces*
Species:	*cerevisiae*

Conclusion regarding the parent and subject strains: Given that the parent and subject strains that are shown by genome sequence analysis to be *S. cerevisiae,* this taxonomic designation is also appropriate for the recipient strain.

B. Donor Microorganisms

The synthetic, codon optimized [redacted] introduced into the subject strains was based on the wild type nucleotide sequence from [redacted] is an obligate chemoautotrophic bacterium and a facultative anaerobic organism capable of respiring aerobically or via denitrification (Beller at al., 2006). This microorganism is

[redacted]

used in water treatment systems for removing [redacted] It naturally expresses [redacted] type [redacted] and [redacted] that convert [redacted] and [redacted] into two molecules of 3-phosphoglycerate.

The synthetic [redacted] gene introduced into the subject strains was based on the [redacted] from [redacted] [redacted]. [redacted] is an edible flowering plant of the Amaranthaceae family. Its leaves are used for human consumption as vegetables. Like other plant photosynthetic organisms, [redacted] fixes [redacted] by converting it to energy-rich molecules such as glucose (Milanez and Mural, 1988).

The synthetic, [redacted] were based on the genome sequence found in [redacted] (Durfee et al., 2008), [redacted] Gram negative, facultatively [redacted] that is capable of both respiratory and fermentative type of metabolism. The typical habitat for [redacted] is the lower intestines of humans and other warm-blooded animals (Kaper et al., 2004).

The synthetic, codon optimized [redacted] was based on the wild type sequence of the same gene found in [redacted], an osmotolerant yeast, is known for its trait to survive in extreme high sugar environment and is characterized by extraordinary adaptation to sugar stress (Dakal et al., 2014). [redacted] also plays a central role in the production of traditional fermented foods, such as [redacted] (Watanabe et al., 2013).

A synthetic, codon-optimized version of the [redacted], introduced into [redacted] strain, was based on the nucleotide sequence from [redacted] is a fungal species within the genus of [redacted] that grows on the undersides of [redacted] wood degradation as their primary means of nutrition (Larsson, 2007).

III. PRODUCTION VOLUME

The subject strains will be manufactured as Active Dried Yeast (ADY) and an alternate form of the product would be the concentrated active modified yeast, referred to as yeast cream. The expected concentration of the yeast is greater than [redacted] colony-forming units (CFU)/g.

According to the submission, the anticipated production volumes for the subject strains [redacted] and [redacted] are as follows:

***Saccharomyces cerevisiae* strain**	**Forecast (kg)**		
	2021	**2022**	**2023**
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

Table 1. Anticipated yields of each *S. cerevisiae* subject strains over the first three years of operation. (Adapted from MCAN sec. 9; *CFUs = colony forming units)

The actual percentage of the total volume allocated to any one strain will be dictated by the market. The commercial product will have approximately the following composition.

Appearance: [redacted]
Yeast dry matter: [redacted]

[redacted]

Water: [redacted]
Sorbitan monostearate (SPAN 60) emulsifying agent: [redacted]
Total yeast count: [redacted]

IV. HISTORY OF USE

As previously mentioned *S. cerevisiae* has a long history of safe use in the baking and brewing industries. It also has a long history of safe use in fuel ethanol production. EPA has reviewed strains of *Saccharomyces cerevisiae* in a number of recent MCANs: [redacted]. In addition, [redacted] MCANs have been strains of *S. cerevisiae* used in [redacted] production [redacted]).

V. CURRENT AND FORESEEN FUTURE USES

The submission states that the subject microorganisms are to be used for industrial ethanol production. The modified *S. cerevisiae* were developed for the purpose of providing robust yeast that could be easily added to the fermentation tanks of fuel ethanol producers. One hundred percent of the microorganisms will go to biofuel ethanol production and there is no other intended, known, or reasonably foreseeable use.

VI. GENETIC MODIFICATIONS

The genetic modifications to arrive at the subject strains [redacted] and [redacted] are described in detail in the Genetic Construction Report (Cameron, 2021).

a. Construction of the Subject Microorganisms

In this MCAN, the submitter has engineered two yeast strains they identify as *S. cerevisiae* [redacted] and *S. cerevisiae* [redacted] which are the subject strains of MCANs [redacted], respectively. Both subject strains were genetically modified to reduce formation of the undesired byproduct [redacted] to the benefit of increasing ethanol yield per molecule of [redacted] sugar released from [redacted]. In addition, strain [redacted] underwent further modifications to yield [redacted] a strain that can produce [redacted] ([redacted] in order to reduce the amount of [redacted] containing [redacted] normally added during the simultaneous [redacted] phase of the ethanol production process (Cameron, 2021).

In summary, Cameron (2021) outlines the intergeneric genes integrated into both subject strains (unless otherwise noted; all were artificially synthesized and codon-optimized for expression in *S. cerevisiae*) as

follows:

- regarding insertion of the pathway:
  -
  -
  -
  -
- regarding the reuptake pathway to maximize the ethanol yield increase by the pathway by providing the pathway with an enlarged cytosolic pool and by conversion of (an undesired byproduct) into ethanol:
  -
  -
  -
- regarding the production of a functional to release only)
  - encoding a from (introduced into strain only and secreted) (MCAN Secs. 4; 5.3);
- plasmids used in strain engineering variants) were removed (MCAN 5.3).

Furthermore, the endogenous gene was deleted from strain but added in at the locus later to make the strain prototrophic for All promoters, terminators, and the complemented gene were amplified from *S. cerevisiae* strain or artificially synthesized without changing the nucleotide sequence of *S. cerevisiae* strain (Cameron, 2021).

In the following figure, Cameron (2021) gives a detailed description of all of the modifications done to create the two subject strains.

[redacted]

VII. CONSTRUCT HAZARD ANALYSIS

A. Inserted Genes

1 [redacted] **encodes** [redacted] **(a.k.a.,** [redacted]

The synthetic sequence based on the [redacted] gene from the chemolithoautotrophic bacterium, [redacted] is codon-optimized for *S. cerevisiae*. The [redacted] gene encodes [redacted] In nature, [redacted] is found in most autotrophic organisms, from diverse prokaryotes to eukaryotic algae and higher plants (Tabita et al., 2008). It is one of the two unique enzymes of the [redacted] pathway, or [redacted]. In this pathway, [redacted] functions to catalyze the actual CO_2 assimilatory (fixation) step to convert [redacted] into [redacted] molecules of [redacted]. Alternatively, [redacted] may act as an internal [redacted] resulting in the formation of one molecule each of [redacted] and [redacted]. These two reactions follow a sequential and ordered mechanism in which enzyme-bound [redacted] is converted to an [redacted] (Hernandez et al., 1996 and references within; Tcherkez et al., 2006). This

[redacted]

enzyme is common in microorganisms and does not pose hazards.

2 [redacted]

For the gene [redacted], encoding [the mature] [redacted]), cDNA from [redacted] [redacted]) (accession number: [redacted] was isolated and PCR-amplified to generate a DNA fragment (Guadalupe-Medina et al., 2013) [redacted] is an essential, light-activated enzyme in most photosynthetic organisms. In [redacted], it is present as a precursor with a transit peptide (~51-56 amino acids) on the N-terminus. The mature form in [redacted] (encoded by the gene used in this submission) is a homodimer that [redacted], the primary [redacted] for [redacted] Subsequently, [redacted] is the substrate for [redacted] (described above). This plant enzyme, located in the chloroplast stroma, is regulated by metabolites, energy charge, and light. Light activation is achieved through [redacted] of the mature protein (Milanez and Mural, 1988; Porter and Hartman, 1988; Porter et al., 1986; and references within). This gene is not expected to pose any hazard.

3. [redacted]

Proteins of the [redacted] class (a subgroup of chaperones) are wide-spread and facilitate the correct three dimensional *de novo* folding or refolding of polypeptides in cellular environments. Two such [redacted] are [redacted] and [redacted] from [redacted] which work together in the crowded conditions of the cell to form catalytically active enzymes on biological timescales and prevent protein aggregation, which could lead to toxic molecular species (Hartl et al., 2011 and references within; Goloubinoff et al., 1989). This system has been studied extensively. As a result of the ubiquity of these [redacted] in nature and their benign activity and specificity for [redacted] these genes are not expected to pose any hazard.

4. [redacted]

The synthetic sequence based on the [redacted] gene from [redacted] is codon-optimized for *S. cerevisiae*. The [redacted] gene encodes [redacted], also known as [redacted] (Kanehisa et al., 2016). [redacted] plays a role in anaerobic [redacted] metabolism and has been isolated from many species of bacteria and is not associated with the production of toxic or otherwise deleterious substances. This enzyme is common in microorganisms. This gene is not expected to pose any hazard.

5. [redacted]

The synthetic sequence based on the [redacted] gene from [redacted] is codon-optimized for *S. cerevisiae*. At the amino acid level, the synthesized and codon-optimized protein here matches 100% of the query to a public version (GenBank [redacted]). Based upon conserved domains, it is a member of the [redacted] subfamily of the Major Facilitator Superfamily of transporters and similar proteins [redacted]). Per Lu et al. (2020) the [redacted] subfamily is comprised of functionally redundant proteins that function mainly in the transport of

[redacted]

[redacted] as well as other [redacted]. As [redacted] transport pathways are found widely in nature, this modification is not expected to present hazard.

[redacted]

The gene coding for [redacted] introduced into the modified strains is a synthetic copy, codon-optimized for *Saccharomyces cerevisiae*. [redacted] is a fungal species within the genus of corticioid fungi that grow on the undersides of dead tree trunks or branches and rely on wood degradation as their primary means of nutrition (Larsson, 2007). For this purpose, they naturally secrete enzymes, including [redacted] also known as [redacted] belongs to family [redacted] in the classification of [redacted] catalyses the release of [redacted] from the non-reducing ends of [redacted] and other [redacted] by hydrolysing termina [redacted] residues. It is secreted from the cell due to the associated signal sequence (Lu et al., 2020). This gene is not expected to pose any in hazard.

7 [redacted]

In addition, each strain contains 2 copies of an artificially synthesized, codon-optimized gene, [redacted] encoding [redacted] from [redacted]. Thus, the subject strains carry their endogenous copies as well as these versions. [redacted] also known as [redacted] or [redacted], uses ATP to phosphorylate [redacted], yielding [redacted], which subsequently feeds into [redacted] and ethanol pathways. This form is composed of a single chain with separable domains homologous to the [redacted] and [redacted] subunits of the [redacted] enzyme, and is found in yeasts and other eukaryotes and in some bacteria, including [redacted] (Marchler-Bauer et al., 2017 re: [redacted]; Molin and Blomberg, 2006). This gene is not expected to pose any hazard.

8. **Addition of other intrageneric genes**

[redacted] (was complemented at the [redacted] loci after deletion of endogenous one).

9. **Deletion/Disruption of Endogenous Genes/Loci**

[redacted] gene was deleted. Subsequently, another copy was complemented at the [redacted] loci, disrupting at some of the [redacted]).

[redacted]

[redacted]

[redacted]

10. Construct Hazard Conclusions

None of the introduced genes in this MCAN pose hazards to human health or the environment. Nor do the genes introduced in previous MCANs. All of the cumulative genetic modifications are merely introducing enzymes of metabolic pathways to improve efficiency of ethanol production from [redacted]. The genetic modifications in this MCAN , introducing the [redacted] re-uptake pathway, enhancing the conversion of [redacted], and increasing the breakdown of [redacted] are all to increase ethanol yields

[redacted]

by the subject strains.

B. Potential for Gene Transfer

The potential for horizontal gene transfer (HGT) of the inserted intergeneric genes from the subject microorganisms to other organisms in the environment must be considered if the subject strains were inadvertently released from the manufacturing facility or ethanol plants. The potential for horizontal gene transfer (HGT) of the introduced genetic material to other microorganisms in the environment is likely low as the literature suggests that horizontal gene transfer rates between fungi are low. According to Novo et al. (2009), horizontal gene transfer events in yeasts have only been rarely described, and the events reported have involved acquisition of single bacterial genes (Wei, 2007; Dujon, 2004; Hall et al., 2005). Gojkovic et al. (2004) also report on the acquisition of a bacterial enzyme by *Saccharomyces* which allowed for cell growth in the absence of oxygen. There is one report of the introgression of 23kb from *S. cerevisiae* to *S. paradoxus* (Liti et al., 2006). Another case of gene transfer was revealed through the genome sequencing of a wine yeast strain, *S. cerevisiae* [redacted] that apparently had acquired genetic material from the non-*Saccharomyces* yeast, [redacted] This is a budding yeast that is a major contaminant in wine fermentations (Novo et al., 2009). According to Dujon (2006), HGT in yeasts is rather rare unlike that of bacteria where HGT is of great importance. Dujon (2006) stated that HGT in yeast is numerically limited, where only a few cases (<0.2% of the total gene number) have been reported.

In addition to the low likelihood of horizontal gene transfer in yeast in general, the introduced genes were stably inserted into the chromosome which lessens the potential for HGT. Thus, there is low concern for horizontal gene transfer of the introduced intergeneric genes in [redacted] and [redacted] to other microorganisms in the environment. Even if HGT was to occur from the subject strains to other organisms if inadvertently released to the environment, there would still be low concern. These are common genes found in many microorganisms and are merely intracellular enzymes of metabolic pathways.

VIII. POTENTIAL HUMAN HEALTH HAZARDS

A. Recipient Microorganism Species

As mentioned earlier, the risk assessment of *S. cerevisiae* for the 5(h)(4) Tiered Exemptions Final Risk Assessment for *S. cerevisiae* (https://www.epa.gov/sites/production/files/2015-09/documents/fra002.pdf) concluded that this yeast presents low hazards to human health and the environment given the extensive use of *S. cerevisiae* in the brewing and food industry, its ubiquitous presence in the environment, and its lack of pathogenicity. The concern for human health effects associated with the recipient microorganisms is low. *S. cerevisiae* is not a primary fungal pathogen that causes disease in immunocompetent hosts.

1. General Population

a. Pathogenicity/Toxicity

There are low human health hazards posed by *S. cerevisiae.* This yeast has a long history of safe use in the in baking and brewing, and in industrial fuel ethanol production. The risk assessment of *S. cerevisiae* for the

[redacted]

5(h)4 Tiered Exemption stated that there are low human health hazards associated with this yeast. Members of the genus *Saccharomyces* are ubiquitous in nature. They are geographically widely distributed and have been observed in a broad range of habitats. Thus, humans are likely frequently exposed.

b. Allergenicity

As previously stated, *S. cerevisiae* has a long history of safe use when used as a leavening agent in breads and in fermentation of beer and wine. *S. cerevisiae* is a common component of human diets. The long history of safe use of this yeast in food and industrial applications suggests that it does not pose allergenicity concerns when used in closed system fermentation.

2. Potentially Exposed or Susceptible Subpopulation

a. Pathogenicity/Toxicity

The potential human health effects of the subject microorganism to a potentially exposed or susceptible subpopulation (PESS) must be considered. A [redacted] includes workers and then those with not fully competent immune systems such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapy.

Some strains of *S. cerevisiae* have been shown to cause infection in humans. However, *S. cerevisiae* infections in humans are rare, and occur primarily, although not exclusively, in severely debilitated, traumatized, and immune-deficient patients (McCusker et al., 1994). There are several articles in the literature on *S. cerevisiae* as an emerging pathogen in immunocompromised hosts.

McCullough et al. (1998) investigated the virulence attributes of strains of *S. cerevisiae* known as [redacted] which is used as a probiotic in Europe for the treatment of chronic or recurrent diarrhea. [redacted] is considered to be part of the species, *S. cerevisiae.* [redacted] strains showed intermediate virulence in [redacted] mice compared with both virulent and avirulent strains of *S. cerevisiae.* The authors suggested that caution be advised in the probiotic use of these [redacted] strains in immunocompromised patients.

A review of the literature of *S. cerevisiae* as a human pathogen was done by Murphy and Kavanagh (1999). They found numerous cases of *S. cerevisiae* vaginitis and of oropharyngeal infection, as well as potentially fatal systemic infection in bone marrow transplant patients and those immunocompromised by AIDS and cancer treatment. They stated that pathogenic isolates exhibit the ability to grow at [redacted]°C, produce proteinase, and are capable of pseudohyphal growth. They considered *S. cerevisiae* as an opportunistic pathogen, but one of low virulence.

Hennequin et al. (2000) reported four cases of *S. cerevisiae* fungemia in patients receiving *S. boulardii* as probiotic therapy. All four patients had an indwelling vascular catheter and it was thought that the catheters were contaminated from contaminated surfaces and hands following opening the packets of freeze-dried *S. boulardii.* According to the authors, *S. cerevisiae* fungemia is very rare.

[redacted]

The most comprehensive review of the literature on infections by *S. cerevisiae* was done by Enache-Angoulvant et al. (2005). Of the 91 documented cases of invasive *S. cerevisiae* infections, 54 were *S. cerevisiae* infections and 37 were cases of *S. boulardii* fungemia. An earlier review by Munoz et al. (2005) found that of the 60 cases of fungemia caused by *S. cerevisiae,* [redacted] of those cases were patients that had received *S. boulardii* probiotic preparations and another [redacted] of the cases were in patients in close proximity to patients that had received the *S. boulardii* therapy. Risk factors for *S. boulardii* fungemia with probiotic administration of *S. boulardii* include the patient's immunocompromised state during serious illness, yeast spore contamination of health workers' hands and surfaces, and introduction of live yeast into the bloodstream from contaminated hands to vascular catheter sites (Venugopalan et al., 2010).

Although *S. cerevisiae* can sometimes cause infection in humans, this is rare. *S. cerevisiae* has a long history of safe use in the baking and brewing industries where humans are routinely exposed to the yeast and it is widely consumed. The fact that strains of *S. cerevisiae* referred to as *S. boulardii* are intentionally ingested as a probiotic therapy further suggests the yeast is safe for consumption. *S. cerevisiae* is ubiquitous in the environment as well, so humans, including immunocompromised ones, are commonly exposed.

None of these susceptible subpopulations with compromised immune systems listed above are expected to be exposed to the submission strain from its manufacture or use in ethanol production as both are closed systems. The only concern would be for severely immunocompromised workers in the fermentation facility or ethanol facilities which is unlikely.

B. Subject Microorganism

1. General Population

a. Pathogenicity/Toxicity

There is low concern for pathogenicity of the recipient microorganism and there is low concern for pathogenicity/toxicity arising from the introduced genetic material. No antibiotic resistance genes remain in the production strains, *S. cerevisiae* [redacted] and [redacted] used for ethanol production.

A search in PubMed was performed to confirm that the donor organism's [redacted] integrated genes have no documented pathogenicity or toxicity to humans. Certain strains of [redacted] are pathogenic to humans, however the genes associated with the pathogenicity of the bacteria are not used in the submission microorganisms.

The subject strains [redacted] and [redacted] do not contain any introduced antibiotic resistance genes, so there is no concern for horizontal gene transfer of antibiotic resistance genes to other microorganisms if inadvertently released to the environment. The absence of the antibiotic resistance genes was verified through antibiotic susceptibility testing and by genome sequencing.

b. Allergenicity

[redacted] is known to be an allergen in the baking industry (Simonis et al., 2014). However, this is due

[redacted]

to repeated exposure to the enzyme in powder form. For production of these *S. cerevisiae* strains, there is a low risk of allergy because (1) the company uses personal protective equipment (PPE), and (2) minimal amount of aerosols are generated during manufacture of the yeast biomass or use in ethanol production facilities as both processes are liquid fermentations in closed system fermentors. The manufacture of the yeast strains occurs in broth culture with little potential for dust production which alleviates concern for respiratory sensitization. Likewise, the use of the yeast strains in ethanol production also occurs in liquid fermentation in closed systems.

Protein sequence homology was compared with known allergens for the protein products of the following genetic inserts using the allergenonline database. A sliding window of [redacted] amino acids was used to identify sequence identities of [redacted] which is the conservative criterion recommend by the WHO/FAO and EFSA for allergenicity/IgE-cross reactivity (FAO/WHO, 2001; EFSA, 2010).

Altogether, sequence homology screens for the submission microorganisms do not suggest that the proteins are allergenic. There is low concern for allergenicity of the subject microorganism.

None of the other introduced enzymes pose allergenicity concerns in humans. They are merely metabolic pathway genes in the cells for increased ethanol production efficiency.

2. **Potentially Exposed or Susceptible Subpopulation**

a. Pathogenicity/Toxicity

Potential human health effects of the subject microorganisms to a potentially exposed (workers) or susceptible subpopulation (immunocompromised individuals) must be considered. As previously stated, *S. cerevisiae* has a long history of safe use in the brewing and baking industries, as well as in fuel ethanol production.

The introduced genetic material do not pose increased pathogenicity or toxicity concerns for potentially exposed or immunocompromised individuals over that of the recipient microorganism species. In addition, no immunocompromised individuals are expected to be exposed to the subject strains from its manufacture or its use in ethanol production.

b. Allergenicity

Allergenicity of the microorganism and the enzymes to the potentially exposed subpopulation, i.e., workers, must be considered. However, good laboratory practices (including safety glasses, gloves, and masks) would result in a low level of exposure and lessen the potential for adverse effects in workers. The long history of safe use in enzyme production suggests that the allergenicity to workers is not a concern.

In terms of allergenicity, the susceptible subpopulation of atopic individuals, those with a genetic predisposition to develop hypersensitivity reactions to environmental antigens, must be considered. However, atopic individuals are not expected to be exposed to the microorganism or the intercellular enzymes as the yeast is intended for industrial manufacture of ethanol in closed system fermentation.

[redacted]

IX. ECOLOGICAL HAZARDS

A. Recipient Microorganism

The recipient microorganism, *S cerevisiae*, does not pose pathogenicity/toxicity concerns to plants or animals. *S. cerevisiae* is a benign yeast with a long history of safe use that is ubiquitous in the environment. The risk assessment of *S. cerevisiae* for the 5(h)4 Tiered Exemption stated that there are low ecological hazards associated with this microorganism.

Although *S. cerevisiae* is associated with human activity from bread baking and fermentation of alcoholic beverages, *S. cerevisiae* is also widespread in nature. It has been recovered from a variety of sites such as soils, sediments, and plant material under different ecological conditions. *S. cerevisiae* is frequently recovered from fresh fruits and vegetables, generally those fruits with high levels of fermentable sugars. In the environment, yeasts can be dispersed by insects, particularly fruit flies (Gilbert, 1980). More recent work by Becher et al. (2012) found that it is the yeast present on fruits that attracts [redacted] (fruit flies), not the fruit volatiles. The presence of yeast on fruit actually supports development of the fruit fly larvae. Apparently, many insects including beetles, flies, ants, and bees interact with yeast in the environment and are likely involved in their transport (Becher et al., 2012).

B. Submission Microorganism

The introduction of any of the synthetic codon-optimized genes does not pose any concerns for pathogenicity/toxicity of the submission microorganisms to plants or animals. Homologs to genes are common in the environment and they are part of a metabolic pathway that only increases the ethanol-producing abilities of the strains.

[redacted] do not pose any concerns for pathogenicity/toxicity of the submission microorganisms to plants or animals. These proteins and enzymes introduce fully functioning [redacted] and [redacted] uptake pathways, which merely reduce the [redacted] production in favor of ethanol production in the subject strains. [redacted] is ecologically ubiquitous and [redacted] and the intermediate products of the utilization of [redacted] are naturally present in all photosynthetic cells. In addition, the integration of the [redacted] or [redacted] into [redacted], ensures this strain can catalyze the release of D-glucose from the non-reducing ends of [redacted] by hydrolyzing terminal [redacted] Although the subject strains may survive if inadvertently released into the environment, there would be no ecological concerns. *S. cerevisiae* is widespread in the environment in sugar-rich niches. The enhanced ethanol formation capability by enabling more efficient [redacted] does not pose ecological concerns.

No antibiotic resistance genes are present in the subject microorganisms, so there is no concern for comprising the therapeutic value of antibiotics used in veterinary medicine or agriculture through the horizontal transfer of these resistance genes to other microorganisms.

[redacted]

X. SURVIVAL

The [redacted] gene from [redacted] may increase the ability of the recipient species to survive on hypersaline and high-sugar environments, as the transport of cellular metabolites has been shown to allow the donor organism to survive in these environments (Dakal et al., 2014). This gene has not been specifically implicated in the sugar and salinity tolerance of the donor species, [redacted], so the relationship between this gene and survival is unclear. Regardless, the recipient species (*S. cerevisiae*) is already able to survive in widespread in the environment and in sugar-rich niches, so this is unlikely to provide a competitive advantage for the recipient organism.

Following the fermentation of the subject strains, they are subjected to a distillation step which involves heating to a range of [redacted] The submitter reports that a [redacted] in cells can be achieved by subjecting the yeast to a temperature of at least [redacted]°C for [redacted] seconds. The wastewater is then subjected to another treatment step designed to achieve a similar degree of cellular inactivation. Bleach is proposed as an attenuation measure in the event of any spills. As a result of these inactivation procedures, environmental releases are not expected to occur except in cases of system failures.

XI. INTEGRATED RISK ASSESSMENT

S. cerevisiae is not pathogenic or toxic to humans, animals, or plants. Commercial strains have a long history of exposure to humans through consumption of yeast-raised breads and other baked goods and consumption of alcoholic beverages. *S. cerevisiae* also has a long history of safe use to workers in the baking and brewing industries. *S. cerevisiae* is also ubiquitous in the environment. The genetic engineering efforts taken to create the two subject strains only resulted in production of more robust *S. cerevisiae* strains with enhanced ethanol production compared to wild-type yeast strains.

The genetic modifications to the recipient *S. cerevisiae* strain pose low concern for human health. The introduced genetic material does not pose pathogenicity, allergenicity, or toxicity concerns. No antibiotic resistance genes are present in the final subject strains. The genetic modifications to the recipient to arrive at the submission strains pose low ecological hazards.

The introduced genes also do not provide a growth advantage relative to the recipient and there is a low probability of releases to the environment.

There are low hazards posed by the use of both subject strains, *S. cerevisiae* strain [redacted] (J-21-0002) and strain [redacted] (J-21-0003), for ethanol production.

[redacted]

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